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(54) PRODUCTION OF NONINFECTIOUS STRUCTURE PARTICLE CONTAINING SURFACE ANTIGEN PROTEIN OF VIRUS BELONGING TO FAMILY FLAVIVIRUS

(57)Abstract:
PURPOSE: To provide a process for the mass-production of live Japanese encephalitis vaccine.
CONSTITUTION: A cell infected with a virus belonging to the family Flavivirus is infected with a recombinant vaccinia virus integrated with a cDNA coding essentially total prM(M) protein originated from a virus of the family Flavivirus and coding essentially total surface antigen protein. The infected cell is cultured to obtain a large amount of non-infectious structure particles.

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* NOTICES *

CLAIMS

[Claim(s)]
[Claim 1] The manufacture technique of the structure grain of the non-infectivity characterized by isolating the structure grain of the non-infectivity containing E protein of the virus which included cDNA of prM (M) protein of the virus origin belonging to the department of a ****** virus which carries out the code of all for all and surface-antigen protein substantially in the cell with which the virus belonging to the department of a ****** virus was infected beforehand, and which it rearranges, and a vaccinia virus is infected and belongs to the department of a ****** virus from a culture supernatant.
[Claim 2] The manufacture technique according to claim 1 that the virus infected beforehand is a ****** 2 type virus.
[Claim 3] The manufacture technique according to claim 1 or 2 which is that to which cDNA included in a vaccinia virus carries out the code of the protein of a Japanese encephalitis virus.
[Claim 4] The claims 1 and 2 whose structure grain of the non-infectivity obtained is the grain not more than sedimentation-coefficient 100S, or the manufacture technique given in three.

[Translation done.]

* NOTICES *

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention] this invention infects the recombination vaccinia virus incorporating cDNA which carries out the code of prM (M) protein and surface-antigen protein (henceforth E protein) of the virus origin belonging to the department of a ****** virus to the cell with which the virus which belongs to the department of a ****** virus beforehand was infected. It is preferably related with the manufacture technique of the structure grain of the non-infectivity characterized by isolating the structure grain of the non-infectivity containing E protein of the virus belonging to the department of a ****** virus from a culture supernatant, and the structure grain of the non-infectivity whose sedimentation coefficient is less than [100S].
[0002]
[Description of the Prior Art] It is asked for the safer and effective vaccine although the attenuated live vaccine of a yellow-fever virus and the vaccine which makes an inactivation Japanese encephalitis virus an active principle are put in practical use as a vaccine of the present ****** virus. For example, into a healthy mouse brain, a present Japanese-B-encephalitis vaccine inoculates a Japanese-encephalitis-virus Beijing stock, and from the mouse whose symptoms were shown, it extracts a brain in sterile, it refines and carries out an inactivation by the alcoholic protamine method, and it has obtained the vaccine undiluted solution (the 2 edition of the revision for National Institute of Health alumni/ae associations "a Japanese vaccine", January 20, Showa 52 Maruzen Co., Ltd. issue).
[0003] In the manufacturing method of such a vaccine, a lot of Japanese encephalitis virus itself was dealt with, and the manufacturing cost was also high for the vaccine manufacture person in charge the top where danger is very high.
[0004] A ****** virus is a virus which made single stranded RNA the genome (mainframe which bears the genetic information of a virus). one polypeptide translated on the basis of this RNA -- the inside of an infected cell -- setting -- SIG -- it being cut by ******** and the protease and becoming ten protein is known Ten protein is C, PrM (M), E and NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 in order (Annu.Rev.Microbiol, 44,649-688 (1990)). Among these protein, prM (M) is divided into preM and M and may be expressed.
[0005] The ****** virus infected with the ****** virus infection cultured cell or the suckling-mouse brain emits ****** thynnine (it abbreviates to SHA below) grain to a non-infectivity out of a cell in addition to a descendant virus.
[0006] A vaccine can also be manufactured using the antigen protein which has the antigenicity of a virus in addition to the virus itself. From such a viewpoint, the method of manufacturing antigen protein with recombinant DNA technique using a prokaryotic cell or an eukaryotic cell is examined. We found out discovering E protein with the sufficient antigenicity to which it rearranges and the process of the virus was carried out correctly which produced especially a vaccinia virus and a baculovirus as a vector (JP,64-74982,A, JP,1-285198,A).
[0007] E protein is protein in which hemagglutinin activity is shown, and since the above-mentioned SHA grain shows hemagglutinin activity, it understands that this grain has E protein. It is the grain of the non-infectivity to which this SHA grain will be emitted with a descendant virus from an infected cell if a ****** virus is infected with a cultured cell or a suckling-mouse brain, and the diameter shows the configuration of the shape of the 14nm shape of a ring, and a doughnut, and the sedimentation coefficient of **** is 70S (the volume "The Toga Viruses" pp 503-529 and on R.W.Schlesinger, Academic Press., Inc., etc.).
[0008] Since the vaccine which made such SHA grain the active principle has structure similar to a virus, having a high antigenicity is expected.
[0009] The little exudation of the structure grain which consists of E protein of the virus which belongs to the department of a ****** virus in the above-mentioned recombination vaccinia virus or the manufacture technique of E protein using rearrange and] the baculovirus is carried out very much at a recombination virus infection cell culture supernatant liquid. However, almost all structures grain will be connoted by the recombination virus infection cell, in order to obtain a lot of grain, it needs the process of an extraction and refining, and its operation is complicated.
[0010] Although this structure grain has hemagglutinin activity like SHA grain and is a non-infectivity, it is unknown whether it is the SHA grain itself at the present which the identification technique of SHA grain has not decided. Then, in this application, SHA grain and the grain which shows the same property are named generically, and it is expressed as SHA Mr. grain. Of course, SHA grain is also contained in SHA Mr. grain.
[0011]
[Problem(s) to be Solved by the Invention] Then, the result which advanced the study zealously aiming at the development of

the technique this invention persons [redacted] the culture supernatant of a recombination vaccinia [redacted] infected cell produce E protein of the virus belonging to the department of a ****** virus in large quantities under such conventional technique or knowledge, By rearranging, if the superinfection of the vaccinia virus is carried out to the cell incorporating cDNA which carries out the code of prM (M) and E protein of a ****** virus with which the ****** virus was infected beforehand It finds out producing the structure grain of the non-infectivity which contains in the culture supernatant E protein of a lot of viruses which essentially belong to the department of a ****** virus, and the grain not more than sedimentation-coefficient 100S which sets E protein to one of the configuration protein preferably, and came to complete this invention.

[0012]

[Means for Solving the Problem] According to this invention, the structure grain of the non-infectivity containing E protein of the virus which it rearranges, and a vaccinia virus is infected, cultivates and essentially belongs to the department of a ****** virus by ultra-centrifugal separation from the culture supernatant incorporating cDNA which carries out the code of the prM(M) E protein to the cell with which the virus belonging to the department of a ****** virus was infected beforehand, and the method of manufacturing the structure grain of a 100 or less-sedimentation coefficient non-infectivity preferably are offered in this way. [of a ******

[0013] As long as it is the virus classified into a vaccinia virus, anything may be used, for example, the virus with which rearranges in this invention and production of a vaccinia virus is presented is the temperature sensitive mutant (refer to the U.S. patent of No. 4,567,147, and JP,62-44178,A) of WR stock (Journal of Virology 49, p.857 (1984)), the Lister stock, and the Lister stock, and New. York Board of Variolation vaccine stocks, such as Health stock and eight stocks of LC16m

[0014] the inside of these viruses -- the pox size on a hatching hen's-egg ****** allantoic membrane -- 3mm or less -- and the propagation impossible temperature in a lagomorph ren cell is suitable for the thing 41 degrees C or less, and as the example, a ****** right [that] stock LA stock given [aforementioned] in JP,62-44178,A and LB stock (CNTM-1-423), eight stocks of aforementioned LC16m, etc. are attenuated nature, and is advantageous in respect of safety

[0015] Moreover, especially if cDNA included in a vaccinia virus in this invention is cDNA of prM (M) protein of the virus which belongs to a ****** virus at least, and E protein which carries out the code of all substantially, it will not be limited, but it can prepare such cDNA using well-known technique (the volumes on "Molecular Cloning" T.Maniatis, Cold Spring Harbor Loboratory Press, (1989)).

[0016] Moreover, although the ****** virus used as the origin of cDNA can be arbitrarily chosen out of the virus belonging to the department of a ****** virus, a Japanese encephalitis virus, a ****** virus, a waist nile virus, etc. are mentioned as the example.

[0017] For example, although cDNA5037 prepared from the Japanese-encephalitis-virus Sagayama stock (the Yale University ****** virus research unit of U.S. Connecticut) is indicated in the example of JP,64-74982,A, in the domain which has the same function substantially with the above-mentioned cDNA in this invention, you may be embellished cDNA (namely, thing in which replaced and inserted and the base sequence carried out the deletion). Of course, it may be embellished by the grade from which an amino acid sequence is different as long as it has the same function substantially.

[0018] Although especially the production technique of the recombination vaccinia virus incorporating cDNA which carries out the code of prM (M) protein and E protein of the virus belonging to the department of a ****** virus is not limited, it is producible by the following technique, for example.

[0019] The first recombination vector by which un-indispensable DNA field was first included in propagation of a vaccinia virus and by which cDNA which carries out the code of the protein of a ****** virus was further inserted in the DNA field is produced. The plasmid pAKJ6 indicated as an example of the first recombination vector in the example of JP,64-74982,A in which cDNA of a Japanese encephalitis virus was inserted, for example is illustrated.

[0020] Subsequently, the code of prM (M) protein and E protein of a virus which belong the promoting agent who acts on the first recombination vector by the vaccinia virus to the department of a ****** virus is carried out. The second recombination vector inserted before cDNA is produced.

[0021] The promoting agent is effective in order to adjust an imprint of cDNA inserted. If cDNA which carries out the code of the prM (M) protein of the virus belonging to a ****** virus to the bottom of rule of the promoting agent whom a vaccinia virus originally has, and cDNA which carries out the code of the E protein are incorporable, it is not necessary to incorporate especially the promoting agent. However, when other, it is desirable to incorporate with cDNA which carries out the code of prM (M) protein and E protein of the virus which belongs the promoting agent to a ****** virus.

[0022] Although the promoting agent who incorporates is not limited especially as long as it functions within a vaccinia virus, he is 7.5K promoting agent's promoting agent who has the activity of 15 times or more preferably especially 10 or more times more than twice preferably. As such promoting agent's example, the following synthetic promoting-agent SL (array number 1) with the synthetic promoting agent of J.Mol.Biol.210 and 771 -784 (1982) publication and the promoting agent about 20 times the activity of 7.5K is mentioned.

[Formula 1]

S r.

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1          10          20          30          40
CTGCAGGTCA ATTCGGTAGT TGCGATATAC ATATTCTGAT
 PstI    50          60          70          80
CACTAATTCC AAACCCAAGC TTTTTTTTTT TTTTTTTTT
        90          100         110         120
GGCATATAAA TAATAAATAC AATAATTAAT TACGCGTCGAC
       late core                          SalI
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[0023] Next introduce the second recombination vector into the animal tissue culture with which the vaccinia virus was infected beforehand, homology recombination is made to cause between vector DNA and viral-genome DNA, and a recombination vaccinia virus is produced. In production of a recombination vaccinia virus, it can carry out according to description of a conventional method (for example, the volume "DNA cloning Vol.IIa practical approach" pp.191-211 and on D.M.Glover, IRL press, Oxford, Washington).

[0024] That is, the second recombination vector is made to introduce into RK13 cell with which the vaccinia virus was infected by the calcium phosphate coprecipitation method, the plaque which the virus ensemble containing the recombination virus obtained is infected with a thymidine-kinase deficit cell, and is grown under BUdR presence is chosen, and it considers as a recombination virus candidate stock. The technique of choosing the virus in which cDNA of a Japanese encephalitis virus was included from these candidates stocks should just carry out plaque purification using the hybridization method which uses this cDNA as a probe.

[0025] Thus, there is the need of infecting the purified virus which rearranges, and belongs a vaccinia virus to this cell at the department of a ****** virus before infecting a recombination vaccinia virus in this invention, a cell and although you make it usually infected with a mammalian cell (henceforth pre-infection). The virus to use will not be limited especially if it is a virus belonging to the department of a ****** virus. As an example, although a ****** virus, a Japanese encephalitis virus, etc. are mentioned, the isolation of the structure grain which the direction with few amounts of the front-infected virus makes the purpose is easy, and the ****** virus with slow propagation of a virus is more suitable from the viewpoint.

[0026] Especially although the mammalian cell used for incubation is not limited especially as long as a ****** virus and a vaccinia virus are infected, its ape kidney cell origin establishment incubation stock Vero cell is desirable.

[0027] After predetermined [pre-infection] time passes, it rearranges further, the superinfection of the vaccinia virus is carried out, and it is made to cultivate under a suitable condition by the conventional method. Although time until it rearranges from pre-infection and it infects a vaccinia virus changes somewhat with viruses used for pre-infection, it is 20 - 36 hours after still preferably 10 - 48 hours after preferably after the 5 hours or more progress of usual.

[0028] It rearranges, without being front-infected, a vaccinia virus will be infected, or it rearranges from pre-infection, and if the time to infection of a vaccinia virus is too short, E protein emitted into a cell supernatant liquid will decrease extremely. Moreover, if time until it rearranges from pre-infection and it infects a vaccinia virus is too long, the problem that refining of structure grain which the amount of production of the front-infected virus increases, and is made into the purpose becomes difficult will be produced.

[0029] Although a preliminary experiment can determine easily the culture condition with a suitable cell after having rearranged into the front-infected cell and infecting a vaccinia virus, it is usually desirable to cultivate incubation temperature at 37 degrees C using the MEM which contains fetal calf serum 5% as a culture medium.

[0030] And structure grain is collected from a culture supernatant by technique, such as ultra-centrifugal separation, after suitable incubation time. the structure grain of the non-infectivity not more than sedimentation-coefficient 100S which contains E protein of a ****** virus if the density gradient centrifugation of the settlings obtained by ultra-centrifugal separation is carried out further -- desirable -- a sedimentation coefficient 70 [about] -- the structure grain (SHA Mr. grain) of the non-infectivity of S can be obtained

[0031]

[Effect of the Invention] the structure grain of the non-infectivity which essentially contains E protein of a ****** virus according to this invention -- desirable -- the structure grain of the non-infectivity not more than sedimentation-coefficient 100S -- further -- desirable -- a sedimentation coefficient 70 [about] -- the structure grain (SHA Mr. grain) of the non-infectivity of S can be obtained It is expected that such structure grain of a non-infectivity can be used as a vaccine.

[0032]

[Example] An example is given to below and this invention is explained to it still concretely.

(Example 1 of reference) Production of the recombination vector (pAKJ6-SL) incorporating cDNA which carries out the code of the promoting agent, Japanese-encephalitis-virus origin prM (M) protein, and the E protein [0033] pAKJ6 (the above-mentioned and the promoting agent are 7.5K promoting agent, and cDNA which carries out the code of the prM(M) E protein is included.) of JP,64-74982,A, the 24th page, and example 1(7) 3 publication is processed by restriction enzymes PstI and SalI, and it is promoting-agent SL given in the array number 1. It inserted instead of 7.5K promoting agent, and the plasmid of about 6.2 Kbps was obtained. [of a Japanese encephalitis virus] It is pAKJ6-SL about this plasmid. It named.

[0034] The synthetic promoting agent's promoting-agent activity included in this plasmid is about 20 times the 7.5K promoting agent.

[0035] (Example 2 of reference) RK-13 cell cultivated by the culture bottle of -- attenuated variola virus stock Production

25cm2 (****** right [that] stock LA[redacted]n in JP,62-44178,A --) of a recombination vaccini[redacted] The propagation impossible temperature of 41 degrees C in a lagomorph kidney cell and the pock size of 2-3mm on a hatching hen's-egg ****** allantoic membrane are inoculated at a rate of 0.1p.f.u. / cell. Recombination plasmid pAKJ6-SL obtained in the example 1 of reference 45 minutes after 10microg is melted in a 2.2ml sterilized water. By Hidaka's et al. (protein, a nucleic acid and an enzyme, 27,340 (1985)) technique, DNA-calcium phosphate coprecipitate was built and the 0.5ml was dropped on the infection RK-13 cell. For 30 minutes, and 37 degrees C and 7%CO2 It put on the incubator gently and 4.5ml of the MEM which contains fetal calf serum 5% was added. Culture medium is exchanged 3 hours [the] after, and they are 48 hours, 37 degrees C, and 7%CO2. The 3 times freeze thawing was cultivated and carried out for every cultured cell within the incubator.

[0036] For recombination field selection, the above-mentioned virus liquid was inoculated into TK-143 cell cultivated by 10cm Petri dish, the laminating of 1% agarose, 5% fetal calf serum, and the 25microg / mlBUdR ** MEM was carried out to it 30 minutes after, and the infected cell was dyed it with neutral red 0.01% after incubation for three days. In order that a virus might be sampled by the Pasteur pipette from the plaque which appeared, this might be suspended in PBS which contains gelatin 2% and a part might carry out a dot hybridization, the spot was carried out to the cellulose membrane and the remainder was saved at -20 degrees C. The membrane which carried out the spot is 1.5M after repeating processing for 5 minutes 3 times with the 1M tris-hydrochloric-acid buffer solution (pH7.0) for 10 minutes by 0.5N sodium hydroxide. It processed for 5 minutes with NaCl and the 0.5M tris-hydrochloric-acid buffer solution (pH7.0). You made it saturated with 2 double SSC (0.15M NaCl, 0.015M sodium citrate), and 80 degrees C was printed for 2 hours.

[0037] 68 degrees C was processed for 2 hours at 4 times SET(0.6M NaCl, 0.08M tris-hydrochloric-acid, 4mM EDTA, (pH7.8))-10 times Denhardt-0.1% SDS. By 4 times SET-10 times Denhardt four to 50 microg [/ml] O [-0.1%SDS-0.1%Na4 P2] denaturation salmon spermium DNA, and the nick translation, by 32P, cDNA of E protein of the Japanese encephalitis virus which carried out the indicator was put in, and the hybridization of the 68 degrees C was carried out for 14 hours.

[0038] The membrane and the X-ray film were piled up after washing, autoradiography was performed, and the spot in which a film carries out a melanism was chosen. The virus liquid corresponding to the spot which carried out the melanism was again inoculated into the cell of TK-143, the laminating of 1% agarose, 5% fetal calf serum, and the 25microg / mlBUdR ** MEM was carried out 30 minutes after, and the infected cell was dyed with neutral red 0.01% after incubation for three days.

[0039] Operation of purification was repeated until it performed the same operation as the above and all the appearing plaques carried out the melanism by the dot hybridization about the plaque which appeared. In this way, the obtained virus is the target recombination vaccinia virus. It is LAJ6-SL about this recombination vaccinia virus. It named.

[0040] (Example 1) Infection hours [24 hours] before the measurement vaccinia virus of E protein in a manifestation and culture supernatant of E protein by the recombination vaccinia virus, the ****** 2 type virus was beforehand infected with Vero cell by m.o.i.2.

[0041] This front-infected Vero cell 4x106 The recombination vaccinia virus (LAJ6-SL) obtained in the example 2 of reference and LAJ6 (refer to the recombination vaccinia virus obtained from pAKJ6 used in the example 1 of reference, JP,64-74982,A, the 24-27th page, and the example 1 (8)) were infected by m.o.i.2, and were cultivated for 18 hours.

[0042] These culture supernatants were filtered with the pore size 0.2micrometer VCF, and 150,000xg and ultra-centrifugal separation of 2 hours were performed. the obtained settlings -- after PBS buffer washing and 10mM carbonic acid buffer (pH9.8) 100microl -- suspending -- further -- this buffer -- by [2 twice] phase dilution -- carrying out -- ELISA plate -- every [well / 50microl/] -- it put, and it was left 4 degree-C all night, and coated

[0043] ELISA was performed for the monoclonal antibody 204 (J. Gen.Virol.67, 2663-2672) diluted 1:10,000 times as a primary antibody, using the alkaline-phosphatase joint anti-mouse IgG (TAGO, Inc.) as a secondary antibody. The amount of manifestations of E protein was measured from the dilution which shows the end point (absorbance 0.1) in ELISA.

[0044] (Example 1 of a comparison) As a Vero cell which was obtained in the example 2 of measurement reference of E protein in a manifestation and culture supernatant of E protein by the recombination vaccinia virus without front infection and which is rearranged and is used for infection of a vaccinia virus, except using what is not being front-infected beforehand, E protein was made to discover and the amount of E protein was measured by the same technique as an example 1.

[0045] (Example 2 of a comparison) When [which obtained the recombination vaccinia virus simultaneously with a ****** 2 type virus in the example 2 of measurement reference of E protein in a manifestation and culture supernatant of E protein at the time of making it infected with a host cell] it rearranged and a vaccinia virus was infected with Vero cell, except infecting a ****** 2 type virus simultaneously (henceforth simultaneous infection), by the same technique as an example 1, E protein was made to discover and the amount of E protein was measured.

[0046] The result of an example 1 and the examples 1 and 2 of a comparison is collected about the dilution which shows the end point (absorbance 0.1) in ELISA, and it is shown in Table 1.

[0047]

[Table 1]

	実施例1	比較例1	比較例2
	前感染有	前感染無	同時感染
LAJ6	4	2	2
LAJ6−S_L	64	4	4

[0048] As compared with the case where simultaneous infection is carried out when the ****** 2 type virus was front-infected and you do not make it front-infected so that clearly from Table 1, incorporating cDNA which carries out the code of cDNA and E protein which carry out the code of the prM (M) protein, it rearranged and it turns out that a vaccinia virus emits E protein out of a cell efficiently. Recombination vaccinia virus LAJ6-SL which has promoting-agent activity stronger than 7.5K promoting agent especially The amount of E protein besides an infected cell increased by leaps and bounds.

[0049] (Example 2) it obtained in the example 2 of refining reference of the grain-like E protein in a culture supernatant -- rearranging -- vaccinia virus LAJ6-SL The culture supernatant of the infected cell was filtered with the pore size 0.2micrometer VCF, and 150,000xg and ultra-centrifugal separation of 2 hours were performed. A settlings is suspended with PBS buffer and they are a 3-30% ******** contest lei (Daiichi Phamaceutical make) and 20mM. Tris-hydrochloric acid (pH8.0), 0.15M The density gradient centrifugation of NaCl was performed on 150,000xg and the conditions of 1 hour. It draws by the constant rate every from the base of an after [centrifugal] centrifugal tube, and ultra-centrifugal separation of each fraction was carried out again, and it was made to sediment after dilution with enough PBS buffers.

[0050] ELISA plate was coated with the settlings of each fraction by the same technique as an example 1, and it checked to which fraction E protein had sedimented in ELISA. The result is shown in drawing 1 . Moreover, when the fractionation of the ****** 2 type virus is carried out by the density gradient centrifugation as control, it is shown in drawing 1 .

[0051] It rearranged from drawing 1 , and E protein in a vaccinia virus infected-cell culture supernatant sedimented later than a ****** virus, namely, the ****** virus showed that it was the grain of a parvus sedimentation coefficient. The sedimentation coefficient of a ****** virus was a parvus thing, and although it was about 130 S, the sedimentation coefficient of the grain-like E protein which sediments later than a virus was about 70 S. In addition, the sedimentation coefficient of a vaccinia virus is still large than a ****** virus.

[0052]

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Field

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TECHNICAL PROBLEM

[Problem(s) to be Solved by the Invention] Then, the result which advanced the study zealously aiming at the development of the technique this invention persons make the culture supernatant of a recombination vaccinia virus infected cell produce E protein of the virus belonging to the department of a ****** virus in large quantities under such conventional technique or knowledge, By rearranging, if the superinfection of the vaccinia virus is carried out to the cell incorporating cDNA which carries out the code of prM (M) and E protein of a ****** virus with which the ****** virus was infected beforehand It finds out producing the structure grain of the non-infectivity which contains in the culture supernatant E protein of a lot of viruses which essentially belong to the department of a ****** virus, and the grain not more than sedimentation-coefficient 100S which sets E protein to one of the configuration protein preferably, and came to complete this invention.

[Translation done.]

* NOTICES *

MEANS

[Means for Solving the Problem] According to this invention, the structure grain of the non-infectivity containing E protein of the virus which it rearranges, and a vaccinia virus is infected, cultivates and essentially belongs to the department of a ****** virus by ultra-centrifugal separation from the culture supernatant incorporating cDNA which carries out the code of the prM(M) E protein to the cell with which the virus belonging to the department of a ****** virus was infected beforehand, and the method of manufacturing the structure grain of a 100 or less-sedimentation coefficient non-infectivity preferably are offered in this way. [of a ******

[0013] As long as it is the virus classified into a vaccinia virus, anything may be used, for example, the virus with which rearranges in this invention and production of a vaccinia virus is presented is the temperature sensitive mutant (refer to the U.S. patent of No. 4,567,147, and JP,62-44178,A) of WR stock (Journal of Virology 49, p.857 (1984)), the Lister stock, and the Lister stock, and New. York Board of Variolation vaccine stocks, such as Health stock and eight stocks of LC16m

[0014] the inside of these viruses -- the pox size on a hatching hen's-egg ****** allantoic membrane -- 3mm or less -- and the propagation impossible temperature in a lagomorph ren cell is suitable for the thing 41 degrees C or less, and as the example, a ****** right [that] stock LA stock given [aforementioned] in JP,62-44178,A and LB stock (CNTM-1-423), eight stocks of aforementioned LC16m, etc. are attenuated nature, and is advantageous in respect of safety

[0015] Moreover, especially if cDNA included in a vaccinia virus in this invention is cDNA of prM (M) protein of the virus which belongs to a ****** virus at least, and E protein which carries out the code of all substantially, it will not be limited, but it can prepare such cDNA using well-known technique (the volumes on "Molecular Cloning" T.Maniatis, Cold Spring Harbor Loboratory Press, (1989)).

[0016] Moreover, although the ****** virus used as the origin of cDNA can be arbitrarily chosen out of the virus belonging to the department of a ****** virus, a Japanese encephalitis virus, a ****** virus, a waist nile virus, etc. are mentioned as the example.

[0017] For example, although cDNA5037 prepared from the Japanese-encephalitis-virus Sagayama stock (the Yale University ****** virus research unit of U.S. Connecticut) is indicated in the example of JP,64-74982,A, in the domain which has the same function substantially with the above-mentioned cDNA in this invention, you may be embellished cDNA (namely, thing in which replaced and inserted and the base sequence carried out the deletion). Of course, it may be embellished by the grade from which an amino acid sequence is different as long as it has the same function substantially.

[0018] Although especially the production technique of the recombination vaccinia virus incorporating cDNA which carries out the code of prM (M) protein and E protein of the virus belonging to the department of a ****** virus is not limited, it is producible by the following technique, for example.

[0019] The first recombination vector by which un-indispensable DNA field was first included in propagation of a vaccinia virus and by which cDNA which carries out the code of the protein of a ****** virus was further inserted in the DNA field is produced. The plasmid pAKJ6 indicated as an example of the first recombination vector in the example of JP,64-74982,A in which cDNA of a Japanese encephalitis virus was inserted, for example is illustrated.

[0020] Subsequently, the code of prM (M) protein and E protein of a virus which belong the promoting agent who acts on the first recombination vector by the vaccinia virus to the department of a ****** virus is carried out. The second recombination vector inserted before cDNA is produced.

[0021] The promoting agent is effective in order to adjust an imprint of cDNA inserted. If cDNA which carries out the code of the prM (M) protein of the virus belonging to a ****** virus to the bottom of rule of the promoting agent whom a vaccinia virus originally has, and cDNA which carries out the code of the E protein are incorporable, it is not necessary to incorporate especially the promoting agent. However, when other, it is desirable to incorporate with cDNA which carries out the code of prM (M) protein and E protein of the virus which belongs the promoting agent to a ****** virus.

[0022] Although the promoting agent who incorporates is not limited especially as long as it functions within a vaccinia virus, he is 7.5K promoting agent's promoting agent who has the activity of 15 times or more preferably especially 10 or more times more than twice preferably. As such promoting agent's example, the following synthetic promoting-agent SL (array number 1) with the synthetic promoting agent of J.Mol.Biol.210 and 771 -784 (1982) publication and the promoting agent about 20 times the activity of 7.5K is mentioned.

[Formula 1]

S r.

```
1          10          20          30          40
CTGCAGGTCA ATTCGGTAGT TGCGATATAC ATATTCTGAT
 PstI   50          60          70          80
CACTAATTCC AAACCCAAGC TTTTTTTTTT TTTTTTTTT
          90         100         110         120
GGCATATAAA TAATAAATAC AATAATTAAT TACGCGTCGAC
       late core                          SalI
```

[0023] Next introduce the second recombination vector into the animal tissue culture with which the vaccinia virus was infected beforehand, homology recombination is made to cause between vector DNA and viral-genome DNA, and a recombination vaccinia virus is produced. In production of a recombination vaccinia virus, it can carry out according to description of a conventional method (for example, the volume "DNA cloning Vol.IIa practical approach" pp.191-211 and on D.M.Glover, IRL press, Oxford, Washington).

[0024] That is, the second recombination vector is made to introduce into RK13 cell with which the vaccinia virus was infected by the calcium phosphate coprecipitation method, the plaque which the virus ensemble containing the recombination virus obtained is infected with a thymidine-kinase deficit cell, and is grown under BUdR presence is chosen, and it considers as a recombination virus candidate stock. The technique of choosing the virus in which cDNA of a Japanese encephalitis virus was included from these candidates stocks should just carry out plaque purification using the hybridization method which uses this cDNA as a probe.

[0025] Thus, there is the need of infecting the purified virus which rearranges, and belongs a vaccinia virus to this cell at the department of a ****** virus before infecting a recombination vaccinia virus in this invention, a cell and although you make it usually infected with a mammalian cell (henceforth pre-infection). The virus to use will not be limited especially if it is a virus belonging to the department of a ****** virus. As an example, although a ****** virus, a Japanese encephalitis virus, etc. are mentioned, the isolation of the structure grain which the direction with few amounts of the front-infected virus makes the purpose is easy, and the ****** virus with slow propagation of a virus is more suitable from the viewpoint.

[0026] Especially although the mammalian cell used for incubation is not limited especially as long as a ****** virus and a vaccinia virus are infected, its ape kidney cell origin establishment incubation stock Vero cell is desirable.

[0027] After predetermined [pre-infection] time passes, it rearranges further, the superinfection of the vaccinia virus is carried out, and it is made to cultivate under a suitable condition by the conventional method. Although time until it rearranges from pre-infection and it infects a vaccinia virus changes somewhat with viruses used for pre-infection, it is 20 - 36 hours after still preferably 10 - 48 hours after preferably after the 5 hours or more progress of usual.

[0028] It rearranges, without being front-infected, a vaccinia virus will be infected, or it rearranges from pre-infection, and if the time to infection of a vaccinia virus is too short, E protein emitted into a cell supernatant liquid will decrease extremely. Moreover, if time until it rearranges from pre-infection and it infects a vaccinia virus is too long, the problem that refining of structure grain which the amount of production of the front-infected virus increases, and is made into the purpose becomes difficult will be produced.

[0029] Although a preliminary experiment can determine easily the culture condition with a suitable cell after having rearranged into the front-infected cell and infecting a vaccinia virus, it is usually desirable to cultivate incubation temperature at 37 degrees C using the MEM which contains fetal calf serum 5% as a culture medium.

[0030] And structure grain is collected from a culture supernatant by technique, such as ultra-centrifugal separation, after suitable incubation time. the structure grain of the non-infectivity not more than sedimentation-coefficient 100S which contains E protein of a ****** virus if the density gradient centrifugation of the settlings obtained by ultra-centrifugal separation is carried out further -- desirable -- a sedimentation coefficient 70 [about] -- the structure grain (SHA Mr. grain) of the non-infectivity of S can be obtained

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[Translation done.]

* NOTICES *

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EXAMPLE

[Example] An example is given to below and this invention is explained to it still concretely.
(Example 1 of reference) Production of the recombination vector (pAKJ6-SL) incorporating cDNA which carries out the code of the promoting agent, Japanese-encephalitis-virus origin prM (M) protein, and the E protein [0033] pAKJ6 (the above-mentioned and the promoting agent are 7.5K promoting agent, and cDNA which carries out the code of the prM(M) E protein is included.) of JP,64-74982,A, the 24th page, and example 1(7) 3 publication is processed by restriction enzymes PstI and SalI, and it is promoting-agent SL given in the array number 1. It inserted instead of 7.5K promoting agent, and the plasmid of about 6.2 Kbps was obtained. [of a Japanese encephalitis virus] It is pAKJ6-SL about this plasmid. It named.
[0034] The synthetic promoting agent's promoting-agent activity included in this plasmid is about 20 times the 7.5K promoting agent.
[0035] (Example 2 of reference) RK-13 cell cultivated by the culture bottle of -- attenuated variola virus stock Production 25cm2 (****** right [that] stock LA given in JP,62-44178,A --) of a recombination vaccinia virus The propagation impossible temperature of 41 degrees C in a lagomorph kidney cell and the pock size of 2-3mm on a hatching hen's-egg ****** allantoic membrane are inoculated at a rate of 0.1p.f.u. / cell. Recombination plasmid pAKJ6-SL obtained in the example 1 of reference 45 minutes after 10microg is melted in a 2.2ml sterilized water. By Hidaka's et al. (protein, a nucleic acid and an enzyme, 27,340 (1985)) technique, DNA-calcium phosphate coprecipitate was built and the 0.5ml was dropped on the infection RK-13 cell. For 30 minutes, and 37 degrees C and 7%CO2 It put on the incubator gently and 4.5ml of the MEM which contains fetal calf serum 5% was added. Culture medium is exchanged 3 hours [the] after, and they are 48 hours, 37 degrees C, and 7%CO2. The 3 times freeze thawing was cultivated and carried out for every cultured cell within the incubator.
[0036] For recombination field selection, the above-mentioned virus liquid was inoculated into TK-143 cell cultivated by 10cm Petri dish, the laminating of 1% agarose, 5% fetal calf serum, and the 25microg / mlBUdR ** MEM was carried out to it 30 minutes after, and the infected cell was dyed it with neutral red 0.01% after incubation for three days. In order that a virus might be sampled by the Pasteur pipette from the plaque which appeared, this might be suspended in PBS which contains gelatin 2% and a part might carry out a dot hybridization, the spot was carried out to the cellulose membrane and the remainder was saved at -20 degrees C. The membrane which carried out the spot is 1.5M after repeating processing for 5 minutes 3 times with the 1M tris-hydrochloric-acid buffer solution (pH7.0) for 10 minutes by 0.5N sodium hydroxide. It processed for 5 minutes with NaCl and the 0.5M tris-hydrochloric-acid buffer solution (pH7.0). You made it saturated with 2 double SSC (0.15M NaCl, 0.015M sodium citrate), and 80 degrees C was printed for 2 hours.
[0037] 68 degrees C was processed for 2 hours at 4 times SET(0.6M NaCl, 0.08M tris-hydrochloric-acid, 4mM EDTA, (pH7.8))-10 times Denhardt-0.1% SDS. By 4 times SET-10 times Denhardt four to 50 microg [/ml] O [-0.1%SDS-0.1%Na4 P2] denaturation salmon spermium DNA, and the nick translation, by 32P, cDNA of E protein of the Japanese encephalitis virus which carried out the indicator was put in, and the hybridization of the 68 degrees C was carried out for 14 hours.
[0038] The membrane and the X-ray film were piled up after washing, autoradiography was performed, and the spot in which a film carries out a melanism was chosen. The virus liquid corresponding to the spot which carried out the melanism was again inoculated into the cell of TK-143, the laminating of 1% agarose, 5% fetal calf serum, and the 25microg / mlBUdR ** MEM was carried out 30 minutes after, and the infected cell was dyed with neutral red 0.01% after incubation for three days.
[0039] Operation of purification was repeated until it performed the same operation as the above and all the appearing plaques carried out the melanism by the dot hybridization about the plaque which appeared. In this way, the obtained virus is the target recombination vaccinia virus. It is LAJ6-SL about this recombination vaccinia virus. It named.
[0040] (Example 1) Infection hours [24 hours] before the measurement vaccinia virus of E protein in a manifestation and culture supernatant of E protein by the recombination vaccinia virus, the ****** 2 type virus was beforehand infected with Vero cell by m.o.i.2.
[0041] This front-infected Vero cell 4x106 The recombination vaccinia virus (LAJ6-SL) obtained in the example 2 of reference and LAJ6 (refer to the recombination vaccinia virus obtained from pAKJ6 used in the example 1 of reference, JP,64-74982,A, the 24-27th page, and the example 1 (8)) were infected by m.o.i.2, and were cultivated for 18 hours.
[0042] These culture supernatants were filtered with the pore size 0.2micrometer VCF, and 150,000xg and ultra-centrifugal separation of 2 hours were performed. the obtained settlings -- after PBS buffer washing and 10mM carbonic acid buffer (pH9.8) 100microl -- suspending -- further -- this buffer -- by [2 twice] phase dilution -- carrying out -- ELISA plate -- every [well / 50microl/] -- it put, and it was left 4 degree-C all night, and coated

[0043] ELISA was performed for the [redacted]oclonal antibody 204 (J. Gen.Virol.67, 2663-2672) [redacted]d 1:10,000 times as a primary antibody, using the alkaline-phosphatase joint anti-mouse IgG (TAGO, Inc.) as a secondary antibody. The amount of manifestations of E protein was measured from the dilution which shows the end point (absorbance 0.1) in ELISA.
[0044] (Example 1 of a comparison) As a Vero cell which was obtained in the example 2 of measurement reference of E protein in a manifestation and culture supernatant of E protein by the recombination vaccinia virus without front infection and which is rearranged and is used for infection of a vaccinia virus, except using what is not being front-infected beforehand, E protein was made to discover and the amount of E protein was measured by the same technique as an example 1.
[0045] (Example 2 of a comparison) When [which obtained the recombination vaccinia virus simultaneously with a ****** 2 type virus in the example 2 of measurement reference of E protein in a manifestation and culture supernatant of E protein at the time of making it infected with a host cell] it rearranged and a vaccinia virus was infected with Vero cell, except infecting a ****** 2 type virus simultaneously (henceforth simultaneous infection), by the same technique as an example 1, E protein was made to discover and the amount of E protein was measured.
[0046] The result of an example 1 and the examples 1 and 2 of a comparison is collected about the dilution which shows the end point (absorbance 0.1) in ELISA, and it is shown in Table 1.
[0047]
[Table 1]

	実施例1	比較例1	比較例2
	前感染有	前感染無	同時感染
LAJ6	4	2	2
LAJ6−S_L	64	4	4

[0048] As compared with the case where simultaneous infection is carried out when the ****** 2 type virus was front-infected and you do not make it front-infected so that clearly from Table 1, incorporating cDNA which carries out the code of cDNA and E protein which carry out the code of the prM (M) protein, it rearranged and it turns out that a vaccinia virus emits E protein out of a cell efficiently. Recombination vaccinia virus LAJ6-SL which has promoting-agent activity stronger than 7.5K promoting agent especially The amount of E protein besides an infected cell increased by leaps and bounds.
[0049] (Example 2) it obtained in the example 2 of refining reference of the grain-like E protein in a culture supernatant -- rearranging -- vaccinia virus LAJ6-SL The culture supernatant of the infected cell was filtered with the pore size 0.2micrometer VCF, and 150,000xg and ultra-centrifugal separation of 2 hours were performed. A settlings is suspended with PBS buffer and they are a 3-30% ******** contest lei (Daiichi Phamaceutical make) and 20mM. Tris-hydrochloric acid (pH8.0), 0.15M The density gradient centrifugation of NaCl was performed on 150,000xg and the conditions of 1 hour. It draws by the constant rate every from the base of an after [centrifugal] centrifugal tube, and ultra-centrifugal separation of each fraction was carried out again, and it was made to sediment after dilution with enough PBS buffers.
[0050] ELISA plate was coated with the settlings of each fraction by the same technique as an example 1, and it checked to which fraction E protein had sedimented in ELISA. The result is shown in drawing 1 . Moreover, when the fractionation of the ****** 2 type virus is carried out by the density gradient centrifugation as control, it is shown in drawing 1 .
[0051] It rearranged from drawing 1 , and E protein in a vaccinia virus infected-cell culture supernatant sedimented later than a ****** virus, namely, the ****** virus showed that it was the grain of a parvus sedimentation coefficient. The sedimentation coefficient of a ****** virus was a parvus thing, and although it was about 130 S, the sedimentation coefficient of the grain-like E protein which sediments later than a virus was about 70 S. In addition, the sedimentation coefficient of a vaccinia virus is still large than a ****** virus.

[Translation done.]

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DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]
[Drawing 1] It is explanatory drawing having shown the distribution pattern of E protein in each fraction which carried out the density gradient centrifugation.

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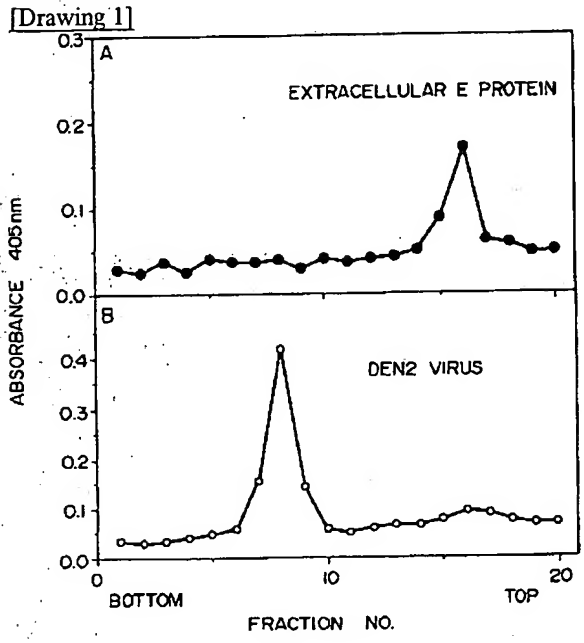
DRAWINGS

[Drawing 1]



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